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(54) Title: RECOMBINANT PLATELET COLLAGEN RECEPTOR GLYCOPROTEIN VI AND ITS PHARMACEUTICAL USE (54) Titre: GLYCOPROTEINE VI DE RECONSTRUCTION DU RECEPTEUR DE COLLAGENE DES PLAQUETTES ET UTILISATION PHARMACEUTIQUE DE CETTE DERNIERE (57) Abstract <p>The invention relates to Glycoprotein VI (GPVI), its isolation, purification, and methods for recombinant production. Especially, the invention relates to the use of GPVI, preferably recombinant GPVI, in the treatment of disorders and pathological events correlated directly or indirectly to blood coagulation disorders such as thrombotic and cardiovascular diseases. The extracellular recombinant protein can also be used for establishing screening assays to find potential inhibitors of the membrane bound GPVI in order to inhibit binding of thrombocytes and platelets, respectively, to collagen. Changes in GPVI can be used to monitor platelet age and exposure to thrombotic and cardiovascular diseases.</p> (57) Abrégé <p>L'invention concerne la glycoprotéine VI (GPVI), son isolation, sa purification et des procédés de production par reconstruction. En particulier, l'invention a pour objet l'utilisation de la GPVI, de préférence la GPVI de reconstruction, dans le traitement de troubles et de problèmes pathologiques liés directement ou indirectement aux troubles de la coagulation sanguine, telles que les maladies cardiovasculaires et les thromboses. La protéine de reconstruction extracellulaire peut également être utilisée dans des dosages pour détecter des inhibiteurs potentiels de la GPVI liée à la membrane pour inhiber la liaison des thrombocytes et des plaquettes, respectivement, au collagène. Les modifications de la GPVI permettent de contrôler l'âge des plaquettes et l'exposition aux maladies cardiovasculaires et aux thromboses.</p>		

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Description

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RECOMBINANT PLATELET COLLAGEN RECEPTOR GLYCOPROTEIN VI AND ITS PHARMACEUTICAL USE

5 The invention relates to Glycoprotein VI (GPVI), its isolation, purification, and methods for recombinant production. Especially, the invention relates to the use of GPVI, preferably recombinant GPVI, in the treatment of disorders and pathological events correlated directly or indirectly to blood coagulation disorders such as thrombotic and cardiovascular diseases. The extracellular recombinant protein can also be used for establishing screening assays to find potential inhibitors of the membrane bound GPVI in order to inhibit interaction of platelets and collagen. GPVI on the platelet surface is modified during the platelet lifetime in vivo and can therefore be used as a marker of the platelet age profile.

15 Glycoprotein VI is a 62/65 kDa (non-reduced/reduced respectively) platelet membrane glycoprotein in which forms a complex together with the Fc γ common subunit. The GPVI subunit contains the collagen binding site and the Fc γ subunit is responsible for signalling. The complex forms one of the major collagen receptors on the platelet surface, critical for platelet activation in response to collagen. The recognition sequence on collagen consists of (GlyProHyp) $_n$ sequences. Patients are known from Japan who have a genetic deficiency of GPVI. They have mild bleeding problems and their platelets respond only weakly to collagen, presumably via other receptors. A great deal has been learned about the signalling cascades originating at GPVI which strongly resemble those from immune receptors including T-cell receptors, B-cell receptors and natural killer cell receptors. These cascades involve src family tyrosine kinases such as Fyn and Lyn as well as p72^{SYK} and many other tyrosine kinases and phosphatases and adaptor proteins such as LAT. A main target of these cascades is activation of phospholipase C γ 2 which splits phospholipids to give the second messengers diacylglycerol and IP $_3$. GPVI is thought to be involved in activation of the platelet integrin α 2 β 1 which has a major role in platelet adhesion to damaged vessel wall. Mice with the Fc γ subunit "knocked-out" have platelets which still show responses to collagen implying that the resting state of α 2 β 1 may also be regulated by the GPVI/Fc γ complex.

5 The platelet collagen receptor GPVI is closely related to the natural killer
activatory receptors of the p58KAR family as well as to Fc α R.

10 The adhesion and activation of resting, circulating platelets at a site of vascular
injury is the first step in a process leading to the formation of a thrombus which is
15 converted into a haemostatic plug. Collagen is one of the major components of
the vessel wall responsible for platelet activation. Many types of collagen exist
and seven of these are found in the subendothelial layers. Several different
20 receptors for collagen have been identified on platelets but the major ones are
now considered to be the integrin $\alpha_2\beta_1$ and the non-integrin GPVI. Although $\alpha_2\beta_1$
is well characterised and both subunits were cloned and sequenced several
years ago, the structure of GPVI has remained elusive although several features
25 have been identified. It was determined about twenty years ago that GPVI is a
major platelet glycoprotein with a molecular mass in the 60-65 kDa range and an
acid pI. Its role as a putative collagen receptor was established following the
15 identification of a patient in Japan with a mild bleeding disorder whose platelets
showed a specific defect of response to collagen and lacked this receptor. This
patient had also developed autoantibodies to the deficient receptor and these
were used to characterise the molecule further. More recently it was established
20 that GPVI is associated non-covalently with the common Fc γ subunit which acts
as the signalling part of the complex. It was also demonstrated that the
recognition sequence on collagen for GPVI is a repeated Gly-Pro-Hyp triplet
within the collagen triple helical structure and that synthetic peptides based on
40 this structure could be used as specific GPVI directed agonists. The GPVI/Fc γ
complex was shown to signal to the platelet interior by an immune receptor-like
25 mechanism, involving activation of p72^{Syk} and leading by a cascade of
kinase/phosphatase/adaptor protein interactions to activation of PLC γ 2 and
hence to release of granules and platelet aggregation. A further step in
45 characterisation of this molecule was the demonstration that the snake C-type
lectin, convulxin, from the Tropical Rattlesnake, *Crotalus durissus terrificus* was
30 able to activate platelets by clustering GPVI through a multimeric interaction.
Convulxin was shown to bind specifically to GPVI providing a method for
purification of this receptor in conjunction with established approaches.

Thus, it is clear from the prior art that GPVI seems to be a very interesting compound in many therapeutical fields above all concerning with applications which are related, directly or indirectly, to blood coagulation events which depend on collagen - platelet interaction. It was, therefore, the goal of the present invention is to provide GPVI in a recombinant form and to show its efficiency as direct therapeutical target or as tool for screening of short compounds, especially chemically synthesized or synthesizable compounds having the capability to inhibit or block the natural platelet-collagen interaction.

The invention relates also to portions or fragments of the GPVI protein which have maintained their biological activity which is the binding to collagen.

The invention was successful in purifying adequate amounts of GPVI for preliminary characterisation and for peptide sequencing. The sequences were used to design primers for PCR to identify a positive sequence in a DNA library. This DNA sequence was then used as a probe to isolate an almost complete cDNA sequence from the library and missing 5'-sequence was obtained using a RACE method from a platelet cDNA library.

The invention was also successful in showing the use of recombinant GPVI as therapeutically applicable compound which is capable, when administered in a patient with e.g. damaged blood vessels, to bind to collagen, thus preventing platelets bearing membrane-bound GPVI from binding to said collagen. The recombinant soluble extracellular domain of GPVI contains the collagen binding site and can prevent platelet activation by collagen. It could therefore be applicable to treatment of disease conditions involving increased platelet activation with collagen, such as atherosclerotic plaque rupture, in diseases such as unstable angina or, during surgical treatment such as Percutaneous Transluminal Coronary Angioplasty (PTCA), where arteries are reopened by inflation of a balloon catheter causing considerable damage to the vessel wall and much platelet activation and often resulting in reclosure of the vessel later. The advantage of recombinant GPVI fragments compared to present treatment methods is that they act at an earlier stage by preventing or reducing platelet activation rather than by suppressing events after platelet activation, such as

5 aggregation by GPIIb-IIIa antagonists. Thus, smaller amounts of platelet granule
contents are released including growth factors and chemokines which are
10 involved not only in wound repair but in the remodelling of the vessel wall by
smooth muscle migration and in attraction of phagocytic cells such as monocytes
5 known to contribute to atherosclerosis. Fab fragment of humanised mouse
monoclonal antibodies against GPVI could be used with similar effect to block
15 GPVI on the platelet surface with similar applications as above.

Recombinant GPVI according to this invention can also be used in a binding
20 assay to collagen to screen for small molecules (in combinatorial libraries for
example) capable of inhibiting this interaction and which can be used to develop
therapeutic compounds which are inhibitors of the collagen-platelet interaction.
By suitable derivatisation these compounds are made orally available. Again the
25 main objective is to prepare compounds reducing GPVI-collagen interactions and
hence platelet activation in situations where platelets come into contact with
15 collagen. The screening technology as such used in this invention is well
established in the prior art. By such screening assays the invention enables
30 finding and developing new targets which can inhibit the natural membrane-
bound GPVI on the platelet surface as a collagen antagonist. Such targets which
20 may be small chemical molecules may then be the basis for further inventions.

Another major application of GPVI and reagents that recognize specific domains
of GPVI is as markers of platelet age and functionality. Young platelets are
40 generally thought to be more active and functional than older ones. Young
platelets bind to and are activated by the snake venom C-type lectin convulxin,
25 which is specific for GPVI, and as they age both the binding and degree of
activation decrease. This can be due to either proteolytic or conformational
45 changes in GPVI or its association with Fcγ due to platelet activation or damage
in the circulation. This can be a useful parameter to measure the age and
30 function profile of platelets in patients as well as in normal persons during medical
controls. The platelet age profile changes in many diseases affecting the bone
50 marrow or the immune system and could be an important diagnostic criterion if
better methods for its determination were available. For example, patients with

5 diseases involving increased platelet turnover will show more young platelets
whereas patients on chemotherapy or radiation treatment will show a steadily
10 aging population. Thus, such an age profile can be used for a precise monitoring
of treatment. In a normal healthy population very little is known about the age
5 profile distribution and its role as a predictor of changes in health. It is not known
whether the changes in GPVI are due to the partial involvement of platelets in
15 haemostatic events and whether changes might be more pronounced in patients
with extensive cardiovascular disease. At present thiazole orange is used to
detect young reticulated platelets containing mRNA. This mRNA soon decays,
20 restricting the method to only the youngest platelets. Reagents which could be
used in such an assay would include GPVI-specific snake venom proteins such
as convulxin, or monoclonal or polyclonal antibodies recognising the N-terminal
region of GPVI or monoclonal antibodies recognising new sites or conformations
25 exposed by proteolysis of the N-terminal domain or specific conformations
present either in the intact molecule and not in the aged one or vice versa or
small chemical entities selected to recognise specifically intact GPVI or its
30 modified form. These reagents would be labelled with a fluorescent marker, or
together with a fluorescent labelled second antibody or affinity reagent and used
in flow cytometry to measure the platelet binding profile. At a later stage
20 alternative, less labour intensive measuring techniques based on automated
measuring of platelet profiles could be adopted. Using cell sorting methods with
35 flow cytometry or magnetic beads it should be possible to isolate young and old
platelets to examine the factors involved in removal of old platelets from the
circulation. Reagents recognizing specific forms of GPVI would be a key to such
40 studies.

Therefore, it is an object of the present invention to provide a DNA coding for
45 Glycoprotein VI or biological active fragments thereof, especially the sequence of
Fig. 2.

30 It is a further object of this invention to provide a DNA coding for Glycoprotein VI
50 comprising the amino acid sequences of Fig. 1a and 1b.

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It is another object of this invention to provide a pharmaceutical composition comprising recombinant GPVI together with a pharmaceutically acceptable diluent, carrier or excipient, and its use for the manufacture of a medicament in the therapeutical field of thrombotic and cardiovascular events and disorders related to platelet-collagen interactions

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Another object of the invention is the use of recombinant GPVI in a screening tool for detecting specific inhibitors of platelet-collagen interactions.

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Another object of the invention is the use of GPVI as a marker for platelet age and exposure to cardiovascular diseases.

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Possible medical indications and applications, respectively, are, for example, unstable angina pectoris, PTCA, use of stents in this field, operations on coronary vessels, general operations on blood vessels, operations which may damage larger blood vessels such as hip joint operations. Moreover, all indications are included which relate to thromboembolic events caused by disorders of the interaction between the vessel wall and the coagulation system with a high risk of formation of thrombi and blocking of vessels.

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As indicated above, the GPVI protein and fragments thereof according to the present invention are suitable as pharmaceutically effective compounds in pharmaceutical compositions and combinations.

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The pharmaceutical formulations according to the invention optionally may comprise additional active ingredients like anti-coagulants such as hirudin or heparin or thrombolytic agents such as plasminogen activator or hementin or antagonists to other platelet receptors such as GPIIb-IIIa antagonists like abciximab or eptifibatide or ADP-receptor antagonists such as clopidogrel.

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The novel protein, and its biological active fragments respectively, according to the invention may form pharmaceutically acceptable salts with any non-toxic, organic or inorganic acid. Inorganic acids are, for example, hydrochloric, hydrobromic, sulphuric or phosphoric acid and acid metal salts such as sodium monohydrogen orthophosphate and potassium hydrogen sulfate. Examples, for

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organic acids are the mono, di and tri carboxylic acids such as acetic, glycolic, lactic, pyruvic, malonic, succinic, glutaric, fumaric, malic, tartaric, citric, ascorbic, maleic, hydroxymaleic, benzoic, hydroxybenzoic, phenylacetic, cinnamic, salicylic and sulfonic acids such as methane sulfonic acid. Salts of the carboxy terminal amino acid moiety include the non-toxic carboxylic acid salts formed with any suitable inorganic or organic bases. These salts include, for example, alkali metals such as sodium and potassium, alkaline earth metals such as calcium and magnesium, light metals of Group IIIA including aluminium, and organic primary, secondary and tertiary amines such as trialkylamines, including triethylamine, procaine, dibenzylamine, 1-ethenamine, N,N'-dibenzylethylene-diamine, dihydroabietylamine and N-alkylpiperidine.

As used herein, the term "pharmaceutically acceptable carrier" means an inert, non toxic solid or liquid filler, diluent or encapsulating material, not reacting adversely with the active compound or with the patient. Suitable, preferably liquid carriers are well known in the art such as sterile water, saline, aqueous dextrose, sugar solutions, ethanol, glycols and oils, including those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil and mineral oil.

The formulations according to the invention may be administered as unit doses containing conventional non-toxic pharmaceutically acceptable carriers, diluents, adjuvants and vehicles which are typical for parenteral administration.

The term "parenteral" includes herein subcutaneous, intravenous, intra-articular and intratracheal injection and infusion techniques. Also other administrations such as oral administration and topical application are suitable. Parenteral compositions and combinations are most preferably administered intravenously either in a bolus form or as a constant fusion according to known procedures. Tablets and capsules for oral administration contain conventional excipients such as binding agents, fillers, diluents, tableting agents, lubricants, disintegrants, and wetting agents. The tablets may be coated according to methods well known in the art.

Oral liquid preparations may be in the form of aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for reconstitution with water or another suitable vehicle before use. Such liquid preparations may contain conventional additives like suspending agents, emulsifying agents, non-aqueous vehicles and preservatives.

Topical applications may be in the form of aqueous or oily suspensions, solutions, emulsions, jellies or preferably emulsion ointments.

Unit doses according to the invention may contain daily required amounts of the protein according to the invention, or sub-multiples thereof to make up the desired dose. The optimum therapeutically acceptable dosage and dose rate for a given patient (mammals, including humans) depends on a variety of factors, such as the activity of the specific active material employed, the age, body weight, general health, sex, diet, time and route of administration, rate of clearance, the object of the treatment, i.e., therapy or prophylaxis and the nature of the thrombotic disease to be treated, antiplatelet or anticoagulant activity.

Therefore, in compositions and combinations useful as anticoagulants in a treated patient (in vivo) a pharmaceutical effective daily dose of the peptides of this invention is between about 0.01 and 100 mg/kg body weight, preferably between 0.1 and 10 mg/kg body weight. According to the application form one single dose may contain between 0.5 and 10 mg of the collagen inhibitor. To achieve an anticoagulant effect in extracorporeal blood a pharmaceutically effective amount of the inventive peptides is between 0.2 and 150 mg/l, preferably between 1mg and 20 mg/l extracorporeal blood.

Short Description of the Figures:

Fig. 1: Protein sequence of GPVI (one-letter-code)

1a: Leader sequence

1b: Mature protein

Open reading frame: 339 amino acids

Asterisk: Glycosylation site
Double underline: Transmembrane domain
Underline: Sequenced peptides

Fig. 2: GPVI nucleotide sequence covering open reading frame of 1017 bp plus 3' and 5' regions total 1249 bp

Detailed description of the invention

Two sequences of 7 amino acids showing the least degeneracy in the genetic code were chosen for the synthesis of DNA primers in order to amplify part of the GPVI cDNA by PCR. As the location of both peptides in the protein were totally unknown, for each of them, two degenerate primers, one sense and one antisense were prepared. These primers were used to amplify a human bone-marrow library. The combination of the sense 5' TYA THC CNG CNA TGA ARMG 3' primer coding for the sequence PAMKRSRL with the antisense 5' TTR TAN ARN GCR AAY TGR TC 3' one corresponding to DQFALYK amplified a DNA fragment of 221 bp. In addition to the selected peptides, the amplified DNA coded for the LysC/AspN peptide DQLELVATGVFAKPSLSAQPGPAVSS, clearly linking the sequence to the cDNA for GPVI.

Screening 600.000 pfu from a bone marrow library with this 221 bp DNA fragment produced 4 positive pfu. Three had inserts of 1350 bp whether cut by the restriction enzymes Sal I or EcoR I and belonged to the IgG superfamily. The fourth one had an 4.6 kb insert by Sal I digestion and gave two fragments of 2300 bp and 1300 bp respectively when treated by EcoRI. Its DNA encoded the sequence for the 10 peptides derived from amino acid sequencing of GPVI but stopped short of the amino terminal. No starting methionine or leader sequence could be found but more than 2000 bp of previously sequenced non-reading frame DNA terminating in an Alu sequence were present. The 5' end RACE experiment was completed on platelet poly A RNA with primers located in a part of the GPVI sequence which had been corroborated by that of the peptides. A fragment of 348 bp including 278 bp on the sequence of the fourth clone and 70 bp new from bp 1987 corresponding to 14 amino acids including the first

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methionine were found before falling back on the established GPVI sequence. Thus, a cDNA containing a total of 1249 bp, a 25 bp 5' sequence upstream of the start codon, an open reading frame of 1017 bp coding for a protein, including leader sequence, with 339 amino acids, and a 3' region of 207 bp including the stop codon could be sequenced.

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A cDNA coding for platelet GPVI was cloned and sequenced from a human bone marrow cDNA library using RACE with platelet mRNA to supply missing 5' sequence. The open reading frame of 1017 bp encodes 339 amino acids and a untranslated 3' region. Hydrophobicity analysis of the amino acid sequence revealed the presence of two putative transmembrane domains, a putative 20 amino acid signal sequence, and a 19 amino acid domain between residues 247 and 265 of the mature protein. The sequence and its amino acid translation are shown in Fig. 2 and Fig. 1. A comparison with the amino acid sequence of the most similar molecules found in a search of GenBank reveals clearly that it belongs to the immunoglobulin superfamily and the extracellular domain contains two Ig C2-domain loops formed by two disulphide bridges. It is a membrane crossing protein class one molecule with the N-terminus at the exterior and traverses the membrane once. The most closely related molecules belong to the natural killer receptor class which contains both inhibitory and activatory types. GPVI clearly belongs to the activatory subclass not only through its function but also because unlike the inhibitory class it does not contain ITIM sequences in its cytoplasmic domain. Neither does it contain any tyrosine residues which might be involved in phosphorylation. There are some threonine and serine residues in this domain but they do not match any criteria for kinase consensus sequences. Like the activatory class of NK receptors, GPVI contains an arginine residue as the third amino acid of the membrane crossing domain which is involved in the complex formation with the Fcγ subunit. The cytoplasmic domain contains 51 amino acids, showing only a minor similarity (in the region just below the membrane) to the cytoplasmic domains of other members of this family. This suggests that this domain in GPVI may associate with different types of cytoplasmic molecule than the other family members. GPVI contains only a single putative N-glycosylation site at Asn69. The domain just above the membrane

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after the beta sheets of the Ig domains finish, however, is rich in threonine and serine residues which could provide O-glycosylation sites such as are found in GPIb α and GPV. The main function of this O-glycosylation seems to be to present the receptor structures well-extended from the platelet surface to facilitate the interactions with their bulky ligands. Since GPVI was earlier established as a sialoglycoprotein, the difference in molecular mass between the theoretical amino acid mass (37 kDa) and the mass determined by gel electrophoresis (65 kDa reduced) must be due to this glycosylation.

10 The structure of natural killer receptors of the two domain type has been established by X-ray crystallographic studies and the two Ig-domains were shown to form an acute angle with the receptor site for the peptide-carrying HLA antigens lying on the outside of the elbow. A direct comparison of the structure of the HLA peptide binding site with that of collagen immediately suggests why these receptors have a common origin because the multiple alpha-helical structures of the HLA binding site and the peptide it contains strongly resemble the triple helical structure of collagen. The natural killer receptors are postulated to work by a dimerisation mechanism with two receptors recognising two separate HLA sites on the cell which the natural killer cell is investigating. Possibly this dimerisation is part of the activation or deactivation mechanism, depending on the class of receptor. In the case of GPVI there may as well be the possibility for two GPVI molecules to associate with one Fc γ , since each monomer of the Fc γ dimer has a recognition sequence. However, the stoichiometry is not yet known, and based upon the structure of collagens, collagen-like peptides that act via GPVI and convulxin, it seems likely that the strength of the signal is related to the number of GPVI/Fc γ complexes that are clustered together. Other platelet receptors belonging to this Ig family include ICAM-2 (CD102) and PECAM (CD31).

30 All microorganisms, cell lines, expression systems, expression hosts, plasmids, promoters, resistance markers, replication origins, restriction sites or other fragments or parts of vectors which are mentioned in the description not directly in connection with the claimed invention are commercially or otherwise generally

available. Provided that no other hints are given, they are used only as examples and are not essential with respect to the invention, and can be replaced by other suitable tools and biological materials, respectively.

- The techniques which are essential according to the invention are described in detail below and above. Other techniques which are not described in detail correspond to known standard methods which are well known to a person skilled in the art, or are described more in detail in the cited references and patent applications and in the standard literature (e.g. Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor; Harlow, Lane, 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor).

EXAMPLES

- Example 1: *Materials*—Protein A-Sepharose, peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies, bovine serum albumin, *Crotalus durissus terrificus* venom, wheat germ agglutinin (WGA), N-hydroxysuccinimidyl chloroformate-activated cross-linked 4% beaded agarose and Triton X-114 were from Sigma Chemical Co. (St Louis, MO), Octanoyl-N-methyl-glucamide (ONMG) and nonanoyl-N-methyl-glucamide (NNMG) were from Oxyl Chemie (Bobingen, Germany).

- Example 2: *GPVI isolation from platelets* — Membrane glycoproteins were isolated from platelets as previously described. Briefly, platelets (from 40 buffy coats) were washed and lysed in 2% Triton X-114 in the presence of protease inhibitors. The Triton X-114 and aqueous phases were separated and the detergent phase was loaded on a column of wheat-germ agglutinin coupled to Sepharose 4B. The platelet glycoproteins were eluted with 10 mM Tris HCl, pH 7.4, 30 mM NaCl, 0.2% octanoyl-N-methylglucamide (ONMG) and 2% N-acetylglucosamine. After dialysis and concentration, the solution of glycoproteins was loaded on a column of concanavalin bound to N-hydroxysuccinimidyl-p-nitrophenyl chloroformate activated cross-linked 4% beaded agarose (1 mg/ml). The column was washed with 4 volumes of 10 mM Tris HCl, pH 7.4, 30 mM NaCl,

0.2% nonanoyl-N-methylglucamide (NNMG), and then with 4 volumes of 10 mM Tris HCl, pH7.4, 30 mM NaCl and 2% NNMG. GPVI was eluted with 0.08% SDS in 10 mM Tris/HCl, pH 7.4. The solution was concentrated and loaded on a preparative gel of 8.5 % polyacrylamide using the Model 491 Prep Cell (BioRad, CA). The preparative electrophoresis was performed under non-reduced conditions following the manufacturer instructions. GPVI eluted as a single band at 65 kDa. The fractions were pooled, concentrated on Centricon-30 (Amicon, Beverly, MA) and resuspended in 10 mM Tris/HCl, pH7.4 and 0.1% ONMG.

Example 3: *Amino acid analysis of GPVI* - GPVI was digested with the endoproteinases LysC and AspN (Boehringer Mannheim, Germany). The 10 peptides generated were separated by reverse-phase HPLC and sequenced on an Applied Biosystem model 477A pulsed-liquid-phase protein sequencer with a model 120A on-line phenylthiohydantoin amino acid analyser.

Example 4: *Amplification of a 221 bp fragment coding for part of GPVI from a λ gt11 cDNA library* -

A sample (10^{10} pfu) (plaque forming units) from a human bone marrow library (Clontech, Palo Alto, CA) was amplified using 2 combinations of 4 degenerate primers. The final primer concentrations were 2 μ M, the dNTP concentration was 200 μ M and 2 U/100 μ l reaction AmpliTaq Gold (Perkin Elmer, Rotkreuz, Switzerland) were used. The PCR conditions were 5 cycles at 37°C followed by 30 cycles at 44°C. The sense 19mer 5' TYATHCCNGCNATGAARMG 3' and the antisense 20mer 5' TTRTANARNGCRAAYTGRTC 3' amplified a 221 bp fragment which was subcloned in Bluescript KS⁺ (Stratagene, La Jolla, CA) and sequenced using the T7 Sequenase kit (Amersham, Switzerland).

Example 5: *Screening the λ gt11 cDNA library with the 221 bp GPVI probe* - The 221 bp fragment was cut from the plasmid, cleaned and labelled with α^{32} P-ATP (20MBq/50 μ l, Hartmann Analytik, Braunschweig, Germany) using the High Prime Labelling kit (Boehringer Mannheim, Switzerland). The human bone marrow library was screened following the manufacturer instructions. Positive phages were grown, their DNA isolated and subcloned in BlueScript using either EcoRI or

Sal I sites and sequenced. Sequencing was performed using the ABS system of RACE- Platelet poly A RNA was prepared as previously described (Power et al., Cytokine 7, 479-482, 1995). Reverse transcription (30 µl) was performed using 5 µg of poly A RNA with the primer 5'TGAATGAGACGGTCAGTTCAGC 3' (20 µM), dNTP (1mM), RNAsin (40 U), 1X AMV buffer and 20 U AMV reverse transcriptase for 20 min at 45°C followed by 20 min at 52°C. The reaction mixture was treated with 2 µl 6N NaOH at 65°C for 30 min, neutralised with 2µl 6N acetic acid, and concentrated in a Centricon 30 (Amicon). An anchor was ligated to the first strand DNA following the protocol of Aptes and Siebert (BioTechniques 15: 890-893, 1993). Nested PCR was performed using a primer complementary to the anchor and the primer 5' TTGTACAGAGCAAATTGGTC 3' (35 cycles, 55°C) and followed by the primer 5' GACCAGAGGCTTCCGTTCTG 3' (30 cycles at 53°C). The highest band (350 bp) was separated by agarose electrophoresis from the lower ones, subcloned into BlueScript, and sequenced.

Example 6: Preparation of anti-GPVI Fab and F(ab')₂-Polyclonal antisera against human GPVI were generated in rabbits. IgG from rabbit anti-GPVI antiserum was purified as described. Digestion of IgG with immobilized papain (Pierce) to generate Fab fragments was performed according to the standard protocol of the supplier. Fab fragments were separated from undigested IgG and Fc fragments using an immobilized Protein A (Sigma) column. The flowthrough was transferred to a dialysis tube, concentrated using solid polyethyleneglycol 20,000, thoroughly dialysed against 20 mM Hepes, 140 mM NaCl, 4 mM KCl, pH 7.4 and stored at 4°C until used. F(ab')₂ fragments were prepared by pepsin digestion of IgG, 1:50 enzyme to substrate ratio (w/w), in 0.5 M acetate buffer, pH 4.0, at 37°C for 18 hr. The pH was corrected to 7.4 with diluted NaOH and the sample was dialysed against 20 mM phosphate, pH 7.4. F(ab')₂ fragments were separated from undigested IgG and Fc fragments using Protein A chromatography. The flow-through was transferred to dialysis tube, concentrated using solid polyethyleneglycol 20 000, intensively dialysed against 20 mM Hepes, 140 mM NaCl, 4 mM KCl, pH 7.4 and stored in aliquots at -20°C. Washed platelets were lysed in Triton X-114 and phase separation was performed on the

soluble material before isolating the membrane glycoproteins associated with the Triton X-114 phase by affinity chromatography on wheat germ agglutinin-Sephadex 4B as described previously. As GPVI represents a very small fraction of the platelet membrane glycoprotein pool, we used the specificity of the snake C-type lectin convulxin for isolation of this receptor. Affinity chromatography on convulxin coupled to Sephadex 4B yielded a 65 kDa protein as major product. However, uncharacterized material of both higher and lower Mr co-eluted with GPVI and could not be removed by extensive washing of the column. Preparative gel electrophoresis on 8.5 % polyacrylamide was added as a final step of purification. Fractions containing GPVI were pooled and gave a single band on reanalysis. Purified GPVI was tested for its ability to block platelet aggregation by collagen. A slight inhibitory effect was observed when aliquots of GPVI solution were added to the platelet suspension. However, by preincubating GPVI with collagen before adding the mixture to the platelet suspension, aggregation could be inhibited in a dose-dependant manner. These platelets still aggregated when fresh collagen was added. Under non-reducing conditions, the isolated protein has a Mr of 62 kDa with a shift toward a slightly higher Mr (65 kDa) under reducing conditions. As the amino terminus of GPVI was found to be blocked, the protein was digested with the enzymes LysC and LysC/AspN which produced 4 and 6 peptides, respectively, from which sequence was obtained. The peptides were separated by reverse phase HPLC on a C4 column and sequenced using the Edman method. The amino acid sequences of these peptides are underlined in the translated cDNA sequence (Fig. 1).

Claims

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Patent claims

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1. DNA coding for Glycoprotein VI or biologically active fragments thereof.

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2. DNA according to claim 1 comprising partially or completely the sequence of Fig. 2.

3. DNA having the sequence of Fig. 2

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4. DNA according to claims 1 – 3 coding for Glycoprotein VI comprising the amino acid sequence of Fig. 1a and 1b.

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5. Recombinant human Glycoprotein VI as medicament comprising the amino acid sequence of Fig. 1b.

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6. Pharmaceutical composition comprising the protein of claim 5 and a pharmaceutically acceptable diluent, carrier or excipient therefor.

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7. Pharmaceutical composition comprising additionally a pharmacologically active compound.

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8. Use of recombinant GPVI in a screening tool for detecting specific inhibitors of platelet-collagen interactions.

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9. Use of recombinant GPVI for the manufacture of a medicament in the therapeutical field of thrombotic and cardiovascular events and disorders related to platelet-collagen interactions.

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10. Use of changes in GPVI as a marker for platelet age and platelet exposure to thrombotic and cardiovascular conditions or events.

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Figure 1.(1a)

1 20
MSPSP TALFC LGLCLGRVPA

(1b)

1
QSGPLPKPSL QALPSSLVPL EKPVTLCQG PPGVDLYRL KLSSSR YQDQ (50)
AVLFIPAMKR SLAGRYCSY QN*GSLWSLPS DQLELVATGV FAKPSLSAQF (100)
GPAVSSGGDV TLQCQTRYGF DQFALYKEGD PAPYKNPERW YRASFPITV (150)
TAAHSGTYRC YSFSSRDPYL WSAPSDPLEL VVIGTSVTPS RLPTEPPSSV (200)
AEFSEATAEL TVSFTNKVFT TETSRITTS PKESDSPAGP ARQYYTKGNL (250)
VRICLGAVID IILAGFLAED WHSARKRLRH RGRAVQRPLP PLPPLPQTRK (300)
SHGGQDGGRRQ DVHSRGLCS (319)

Figure 2:

GAGCTCAGGA CAGGGCTGAG GAACCATGTC TCCATCCCCG ACCGCCCTCT (50)
TCTGTCTTGG GCTGTGTCTG GGGCGTGTGC CAGCGCAGAG TGGACCGCTC (100)
CCCAAGCCCT CCTCCAGGC TCTGCCCAGC TCCCTGGTGC CCCTGGAGAA
GCCAGTGACC CTCGGTGCC AGGGACCTCC GGGCGTGGAC CTGTACCGCC (200)
TGGAGAAGCT GAGTTCCAGC AGGTACCAGG ATCAGGCAGT CCTCTTCATC
CCGGCCATGA AGAGAAGTCT GGTGGACGC TACCGGTGCT CCTACCAGAA (300)
CGGAAGCCTC TGGTCCCTGC CCAGCGACCA GCTGGAGCTC GTTGCCACGG
GAGTTTTTGC CAAACCTCG CTCTCAGCCC AGCCCGGCCG GGGCGTGTGC (400)
TCAGGAGGGG ACCTAACCTT ACAGTGTGAG ACTCGGTATG GCTTTGACCA
ATTTGCTCTG TACAAGGAAG GGGACCTGCG GGCCTACAAG AATCCCGAGA (500)
GATGGTACCG GGCTAGTTTC CCCATCATCA CGGTGACCGC CGCCACAGC
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GGCCCCCAGC GACCCCTGCG AGCTTGTGGT CACAGGAACC TCTGTGACCC
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GACTTCTAGG AGTATCACCA CCACTCCAAA GGAGTCAGAC TCTCCAGCTG (800)
GTCTGCCCCG CCAGTACTAC ACCAAGGGCA ACCTGGTCCG GATATGCCTC
GGGGCTGTGA TCCTAATAAT CTTGGCGGGG TTTCTGGCAG AGGACTGGCA (900)
CAGCCGGAGG AAGCGCCTGC GGCACAGGGG CAGGGCTGTG CAGAGGCCCG
TTCCGCCCTT GCGGCCCTC CCGCAGACCC GGAATCACA CGGGGTGAG (1000)
GATGGAGGCC GACAGGATGT TCACAGCCGC GGGTTATGTT CATGACCGCT
GAACCCAGG CACGGTCGTA TCCAAGGGAG GGATCATGGC ATGGGAGGCG (1100)
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Cys Leu Gly Leu Cys Leu Gly Arg Val Pro Ala Gln Ser Gly Pro Leu
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Pro Lys Pro Ser Leu Gln Ala Leu Pro Ser Ser Leu Val Pro Leu Glu
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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/EP 00/03683

A. CLASSIFICATION OF SUBJECT MATTER		
IPC 7	C12N15/12	C07K14/705 A61K38/17 G01N33/68
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC 7 C07K C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
EPO-Internal, BIOSIS, STRAND, WPI Data, CHEM ABS Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GIBBINS JONATHAN M ET AL: "Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor gamma-chain." FEBS LETTERS, vol. 413, no. 2, 1997, pages 255-259, XP002143941 ISSN: 0014-5793	1
A	the whole document --- -/-	2-9
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document relating to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "B" document member of the same patent family		
Date of the actual completion of the international search		Date of making of the international search report
1 August 2000		21/08/2000
Name and mailing address of the ISA European Patent Office, P.O. 5618 Patentsan 2 NL - 2200 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 report, Fax: (+31-70) 340-3018		Authorized officer Gurdjian, D

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/03683

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JANDROT-PERRUS MARTINE ET AL: "Adhesion and activation of human platelets induced by convulxin involve glycoprotein VI and integrin alpha-2-beta-1." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 43, 1997, pages 27035-27041, XP002143942 ISSN: 0021-9258	1
A	the whole document	2-9
X	GIBBINS JONATHAN M ET AL: "The p85 subunit of phosphatidylinositol 3-kinase associated with the Fc receptor gamma-chain and linker for activator of T cells (LAT) in platelets stimulated by collagen and convulxin." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 51, 18 December 1998 (1998-12-18), pages 34437-34443, XP002143943 ISSN: 0021-9258	1
A	the whole document	2-9
L	WO 95 11259 A (THROMBOSIS RES INST ;JEHANLI AHMED MOHAMMED TAKI (GB); PATEL GEETA) 27 April 1995 (1995-04-27) abstract; claims 1-25	1
P,X	CLEMETSON JEANNINE M ET AL: "The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcalphaR and the natural killer receptors." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 41, 8 October 1999 (1999-10-08), pages 29019-29024, XP002143944 ISSN: 0021-9258	1-5
P,X	the whole document	
P,X	MIURA Y: "Platelet glycoprotein VI" EMBL DATABASE ; ACCESSION NUMBER AB035073, 14 January 2000 (2000-01-14), XP002143945 the whole document	1-5

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No
PCT/EP 00/03683

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